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Our approach is based on the hypothesis that genetic anticipation occurs in some breast cancer families, and is characterized by a decrease in age of onset of the disease in successive generations of affected family members. Our objective is to identify putative breast cancer predisposition gene(s) which have undergone intergenerational expansions of trinucleotide repeats during germline transmission from mother to daughter using the Rapid Expansion Detection System. Recent investigations in other genetic diseases have revealed that anticipation is the result of expansion of trinucleotide repeats in disease susceptibility genes. We have successfully established the system for fragmentation of the digested DNA and its extraction from the gel slices. This provides better resolution of the repeats in the genome. Ligation reaction in the presence of a designed positive Androgen Receptor control sample has been optimized and vertical gel separation conditions have been established. Currently we have been optimizing the method to increase the signal detection of the repeats. Our approach has the potential to allow the rapid identification of novel breast cancer predisposition genes which will provide obvious benefits for families with breast cancer, as well as the potential for insights into the pathobiology of this devastating disease.

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INTRODUCTION

Genetic factors have been shown to influence the risk of developing breast cancer. To date only two dominant predisposition genes, BRCA1 and BRCA2, have been identified and shown to confer a high risk of developing breast cancer when mutated in the germline (Miki et al 1994, Wooster et al., 1995, Tavigtian et al., 1996). However, there is now clear evidence that other important predisposition genes contribute to the susceptibility to breast cancer (Serova et al. 1997). Classic genetic approaches to identify susceptibility loci involve whole genome scans by linkage analysis. These studies are labor-intensive, and require extended families for success. Our approach is based on the hypothesis that genetic anticipation occurs in some breast cancer families, and is characterized by a decrease in age of onset of the disease in successive generations of affected family members. Recent investigations in other genetic diseases have revealed that anticipation is the result of intergenerational instability of trinucleotide repeats in disease susceptibility genes (Sanjeeva et. al., 1997). Importantly, the typical expansion that occur are readily detected by the Repeat Expansion Detection (RED) system, thus bypassing the traditional search for predisposition genes. Our objective, therefore, is to identify putative breast cancer predisposition gene(s) which have undergone intergenerational expansions of trinucleotide repeats during germline transmission from mother to daughter. We will utilize the BRCA1 and BRCA2 mutation negative families that we have accrued, and study mother-daughter pairs with a significant decrease in the age of breast cancer onset from one generation to the next. Our approach has the potential to allow the rapid identification of novel breast cancer predisposition genes which will provide obvious benefits for families with breast cancer, as well as the potential for insights into the pathobiology of this devastating disease.

Statement of Work

Technical Objective 1:	Identification of trinucleotide repeat expansion in mother daughter-pairs with breast cancer.
Task 1: Months 1-3	The optimization of the RED analysis on control samples.

Task 2: Months 2-14	Digestion of DNA samples with EcoR1 and preparation of gel fragments from mother-daughter pairs as well as from control specimens. The RED analysis will be carried out for CAG/GTC trinucleotide repeat.
Task 3: Months 12-24	RED analysis for the CGG/GCC repeat.

BODY - RESULTS

Our initial goal was to develop the most efficient approach for trinucleotide repeat detection before actually screening patient DNA samples. Increasing the efficiency is very important for the proposed RED method since the method utilizes a linear as opposed to exponential amplification as would be in a regular PCR reaction. Unfortunately, this necessitates the use of high quantities of starting genomic template from the patient samples. Since patient DNA material is a limited source we specifically interested to develop the most efficient approach. We have been doing so taking into consideration the following steps.

Genomic DNA digestion and fragmentation

Optimization of genomic DNA digestion has been performed using cell lines established from normal as well as breast adenocarcinoma tissue. Various concentration of cell line DNA was cut using differential concentrations of the digestion enzymes. As a result we have found that the digestion of 20ug of genomic DNA was successfully done using 20 Units of EcoR1 enzyme (Amersham) after incubating the mixture overnight at 37^0C. We also found that dilution of the genomic DNA and addition of extra 20 Units of Ecor1 after several hours enhanced the efficiency of digestion. Then the digested DNA was concentrated using the speed vacuum apparatus. The quality of digestion (complete versus partial digestion) was checked by agarose gel electrophoresis.

Given the number of repeat sites in the genome, analysis of total genomic DNA can complicate detection of expansion of specific repeats. Alternatively, total genomic DNA can be divided into smaller fractions to simplify the analysis and facilitate result interpretation (Koob et al. 1998). The digestions were run on a 1% agarose gel in 1X TBE buffer at 50

Volts for 16 hours (Figure 1, left panel). The lanes containing the genomic digestions were cut from the gel and each lane was sliced into fragments of 0.5cm length each. The DNA was extracted for each fragment using QiaQuick gel extraction kit (Qiagen). The extracted DNA fragments were checked on a 1% agarose gel (Figure 1, right panel). The extractions were stored in -20^0C to be used for ligation.

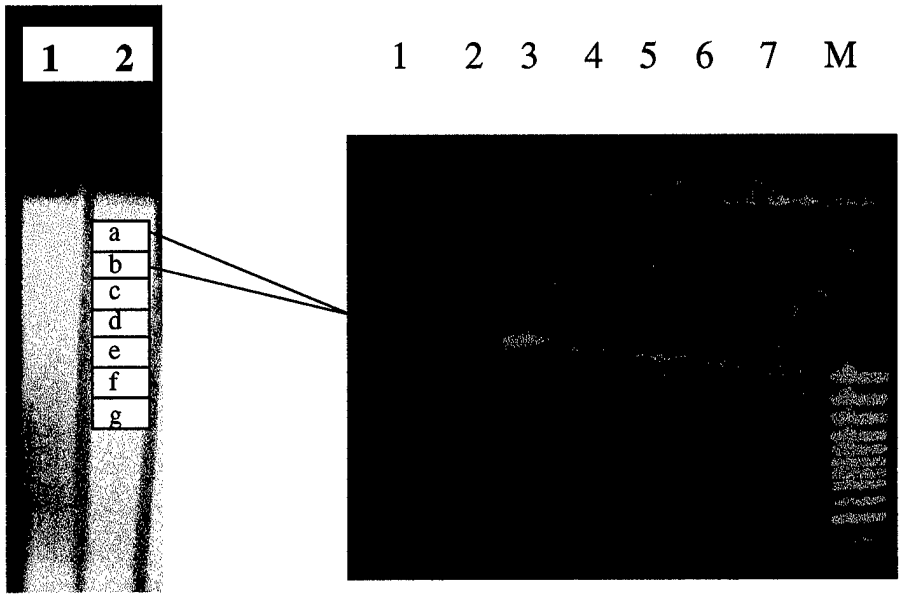
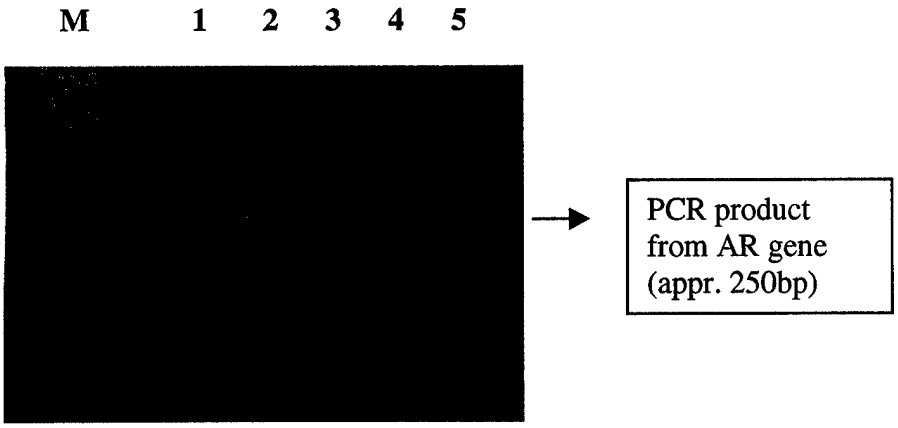


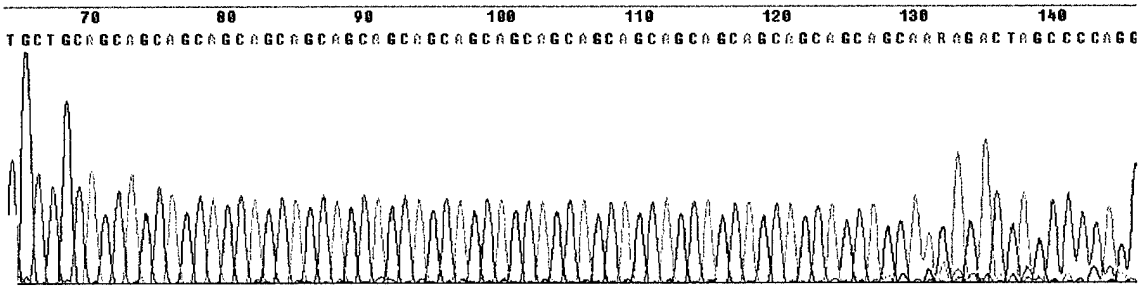
Figure 1: The digested genomic DNA from two samples run on 1% agarose gel (left panel) and the DNA extracted form the gel slices (right panel) lane 1-7 contains the DNA extracted form slices a-f and lane 8 contains a size marker.

Designing control template containing trinucleotide repeats

To monitor the ligation efficiency, it is important to use control PCR products containing trinucleotide repeats. For this purpose we have designed primers to amplify exon1 of the Androgen Receptor (AR) gene which contains a highly polymorphic

trinucleotide (CAG) repeat region (Figure 2). This PCR product was used as a positive control for each ligation experiment to ensure the efficiency of the ligation reaction.





CAG repeat region

Figure 2: PCR products of CAG repeat region in the androgen receptor gene run on agarose gel showing different size CAG repeat containing products from different individuals (top panel), and the sequencing of the fragment from a single indicidual to show the presence of CAG repeat region (bottom panel).

Repeat Expansion Detection (RED) – Ligation reaction

The RED technique is used to detect repeat expansion in DNA (Schalling et al., 1993). Trinucleotide repeat-specific oligonucleotides are annealed to genomic repeat sequences. In the presence of a thermostable ligase, the DNA bound oligonuleotides are ligated together, forming longer oligonucleotide units, which are reflective of the length of specific repeats in the genomic DNA.

For optimizing the ligation reaction we started with the 51 basepair of synthetic oligonucleotide comprised of 17 CTG repeats ((CTG)17 oligo). Inorder to be able to ligate by the ligase, oligonucleotides was first phosphorylated using T4 polynucleotide kinase (Epicenter biotechnology). Ligation reactions were performed in 20ul volume containing 1X Ampligase reaction buffer, the DNA fragments extracted from the gel cuts (about 0.5ug), 50-200ng phosphorylated oligo(CTG)17, and 10-20units of Ampligase enzyme (Epicenter technology). The following cycling conditions were used to cycle the ligation reaction using a 9600 GeneAmp PCR thermal cycler (Perkin Elmer): 94^0C for 20'';70^0C for 30'' repeated for 400 cycles.

In order to increase the efficiency of repeat detection we have performed several experiments trying out different conditions testing the effect of varying genomic DNA concentration, ligase enzyme concentration and the thermal cycling conditions.

Repeat Expansion Detection (RED) – Vertical Gel Electrophoresis

After the ligation reaction the RED products are electrophoresed on urea-containing polyacrylamide gels to separate the RED products according to their molecular weight. For this purpose, the ligation reaction products were mixed with loading buffer containing 50% formamide, denatured by heating at 95^0C for 5 minutes and were run on 6% denaturing polyacrylamide gel at 1500 volts for 1 hour. For each set of reactions we perform a short and a long electrophoresis (1 to 3 hours) to ensure that both long and short repeats will be detected at a high resolution.

Repeat Expansion Detection (RED) – Hybridization by Labelled Oligos

In order to be able to detect the ligation products, the acrylamide gels are blotted onto positively charged nylon membranes (Amersham) by capillary blotting. The ligation products on membranes are immobilized by UV irradiation. The signal detection is carried

out by hybridization of the membranes with CAG repeat containing probes end-labelled with ^{32}P ATP. The membranes were exposed to X-ray film (kodak) for 2-5 days.

Detection of satisfactory signal intensity using this approach has been a very challenging in our study. In order to increase the signal detection we have carried out many experiments using necessary controls at each step. We have designed experiments to test the effect of blotting time on the signal detection. Since hybridization temperature is a critical parameter, we also designed several experiments to test a range of hybridization temperature (50-65^{0}C), as well as hybridization times. Experiments are also carried out using different concentrations of end-labelled oligo probes.

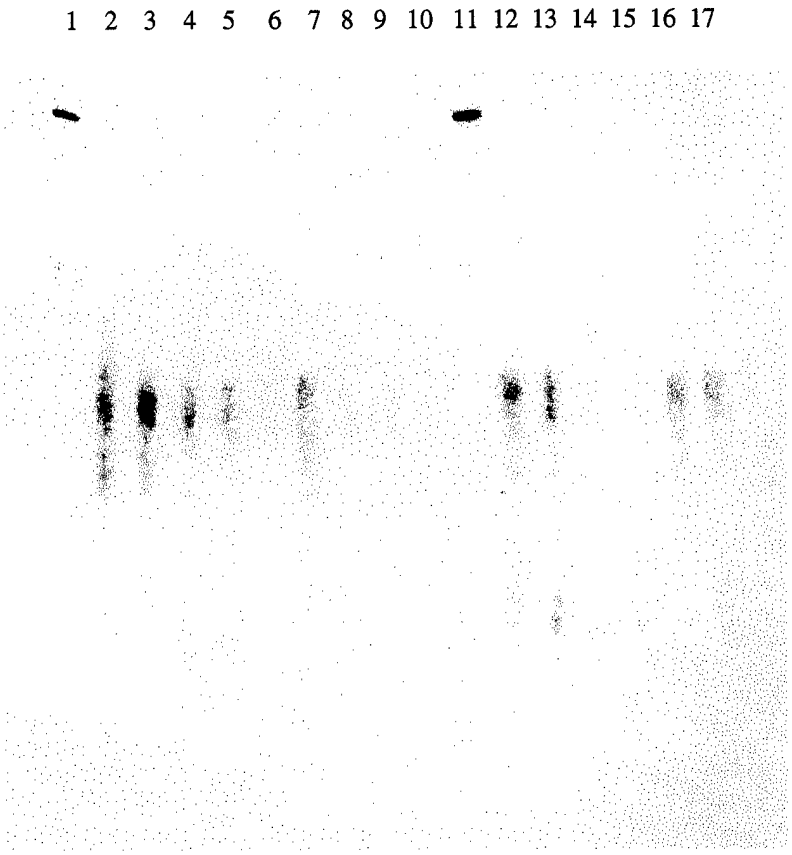


Figure 3: Autoradiogram of ligation products. Lanes 1 and 11 contain PCR products of the CAG repeat region of the Androgen receptor gene. Other lanes contain the ligation reaction products.

Using the hybridization procedure we were not able to detect satisfactory signals on the ligation products (Figure 3). During this period we were able to make sure that every step of this procedure has been checked for quality control and that the unsatisfactory results were not due to an experimental mistake. Throughout optimization we have used the PCR product from AR gene as a positive control for quality check. Since AR PCR product contains approximately 50 CAG repeat, it binds the end-labelled oligo demonstrating a signal (250bp) on the films (Figure 3). As can be seen from the figure, positive signal obtained for AR control sample whereas the intensity for the cell line specimens were very low. At this point we were able to conclude that the low signal obtained in specimens was due to the low copy number of ligation products on the membrane. This is unfortunately due to fact that the RED method utilizes a linear instead of exponential amplification of genomic DNA, thus yielding limited copy numbers of ligation products. Additionally, there is a considerable amount of loss during the transferring of these products onto the membranes. We are currently at the stage to improve the detection of signal intensity by modifying the system as following a) by labeling the ligation products during ligation reaction b) by using PCR generated hybridization probes incorporated with labeled nucleotides

a) Labeling During Ligation

Since we strongly believe that transfer onto membranes can result in loss of ligation products, we have eliminated the transfer and the hybridization step. In this approach we have used end-labelled oligos during the ligation reaction. The (CTG)17 oligos were end labelled with ^{32}P and added into the ligation reaction, incorporating into the ligation products directly. The resulted ligation products are now labelled thus elimination an additional hybridization step using labelled probes. In this case the products are run on polyacrylamide gels and instead of transfer, they are dried at 80^0C using gel dryer apparatus. The gels are exposed to X-ray film. We are currently trying out several conditions to increase the signal intensity using this approach.

b) Using PCR generated hybridization probes incorporated with labeled nucleotides

As described above the RED approach uses a linear amplification protocol thus yielding limited amount of ligation products using genomic DNA as a template. Since we cannot further increase the genomic DNA used, we decided to use probes that might be much more efficient than end-labelled oligo probes. For this reason we designed primers to amplify CAG regions from genomic DNA which contains at least 40 CAG repeats using labelled nucleotides. Since the PCR product will incorporate the labelled nucleotides into the CAG containing products they will be labelled at several nucleotides. These PCR probes will provide more efficient labelling capacity compared to end labelled oligo probes. We are currently trying to improve the signal detection using this approach.

CONCLUSION

The major effort in the first year of the grant has been dedicated to optimize the approach to be able to increase the efficiency of screening for expansion of trinucleotide repeats. We have successfully established the fragmentation of the digested DNA and its extraction from the gel slices. We have also shown that the digested DNA (extracted or in the gel slice) when frozen stays intact for future experiments. Genomic DNA from several patients mother-daughter has been digested, sliced from gels, extracted and stored. We have designed a positive CAG containing control sample using Androgen Receptor gene as a template. This will help to maintain the consistency of the screening. Detection of RED products was achieved for control samples but obtained signals were not strong enough to be used in screening patient mother-daughter DNA. We are currently working on the final part of optimizing the signal detection of RED products. The alternative RED approaches that uses incorporated labeling seem to be promising in enhancing the sensitivity of the system. Shortly we are planning to start screening the patient mother-daughter DNA pairs for the presence of trinucleotide repeat expansion.

Red Detection System has the potential to identify trinucleotide repeat expansion in genes, functions of which are very important for the normal cell function. Discovery of such genes will provide valuable knowledge for understanding breast cancer.

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